Final Scientific Report

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BARD Project Number: IS-3539-04

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Project Title: Diagnostic, Eco-Epidemiology and Control of KHV, A New Viral Pathogen of Koi and Common Carp.

Investigators	Affiliated Institutions
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Keywords: Koi herpesvirus, cyprinid herpesvirus, genome sequence, gene expression, vaccine, serology, fish virus detection

Abbreviations commonly used in the report, in alphabetical order**:**

CNGV – carp interstitial nephritis and gill necrosis virus, aka KHV, CyHV-3
CyHV-1 – cyprinid herpesvirus type 1
CyHV-3 – cyprinid herpesvirus type 3, aka KHV and CNGV
ELISA – enzyme linked immunosorbent assay
KHV – koi herpesvirus
PCR – polymerase chain reaction
TK – thymidine kinase

Budget: IS: $ 150,000 US: $ 140,000 Total: $ 290,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	3	3		6
Submitted, in review, in preparation	2			2
Invited review papers		1		1
Book chapters				
Books				
Master theses				
Ph.D. theses			1	
Abstracts				
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Dr. Myriam Ravins (HUJ)

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		2		2
Longer Visits (Sabbaticals)				

Description Cooperation:
For the field validation of diagnostic tests (PCR), we have exchanged methods and tested in both laboratories independently the developed methods. The TK PCR developed at HU is being used and tested also now at Davis. This test is now for the OIE the reference assay for the diagnosis of KHV infection. Likewise, the enzyme linked immunosorbent assay (ELISA) test developed at Davis was transferred to HU and we are now using it and evaluating it in Israel. The evaluation of new sensitive tools such as LC-PCR/TaqMan PCR has been done on combined samples from Israel and the US.

Patent Summary
None

Abstract
Original objectives and revisions-The proposed research included these original objectives: field validation of diagnostic tests (PCR), the development and evaluation of new sensitive tools (LC-PCR/TaqMan PCR, antibody detection by ELISA) including their use to study the ecology and the epidemiology of KHV (virus distribution in the environment and native cyprinids) and the carrier status of fish exposed experimentally or naturally to KHV (sites of virus replication and potential persistence or latency). In the course of the study we completed the genome sequence of KHV and developed a DNA array to study the expression of KHV genes in different conditions.
Background to the topics-Mass mortality of koi or common carp has been observed in Israel, USA, Europe and Asia. These outbreaks have reduced exports of koi from Israel and have created fear about production, import, and movements of koi and have raised concerns about potential impacts on native cyprinid populations in the U.S.A.
Major conclusions-A suite of new diagnostic tools was developed that included 3 PCR assays for detection of KHV DNA in cell culture and fish tissues and an ELISA assay capable of detecting anti-KHV antibodies in the serum of koi and common carp. The TK PCR assay developed during the grant has become an internationally accepted gold standard for detection of viral DNA. Additionally, the ELISA developed for detecting serum anti-KHV antibodies is now in wide use as a major nonlethal screening tool for evaluating virus status of koi and common carp populations. Real time PCR assays have been able to detect viral DNA in the internal organs of survivors of natural and wild type vaccine exposures at 1 and 10^3 genome equivalents at 7 months after exposure. In addition, vaccinated fish were able to transmit the virus to naive fish. Potential control utilizing hybrids of goldfish and common carp for production demonstrated they were considerably more resistant than pure common carp or koi to both KHV (CyHV-3). There was no evidence that goldfish or other tested endemic cyprinids species were susceptible to KHV. The complete genomic sequencing of 3 strains from Japan, the USA, and Israel revealed a 295 kbp genome containing a 22 kbp terminal direct repeat encoding clear gene homologs to other fish herpesviruses in the family *Herpesviridae*. The genome encodes156 unique protein-coding genes, eight of which are duplicated in the terminal repeat. Four to seven genes are fragmented and the loss of these genes may be associated with the high virulence of the virus. Viral gene expression was studies by a newly developed chip which has allowed verification of transcription of most all hypothetical genes (ORFs) as well as their kinetics.
Implications, both scientific and agricultural- The results from this study have immediate application for the control and management of KHV. The proposal provides elements key to disease management with improved diagnostic tools. Studies on the ecology of the virus also provide insights into management of the virus at the farms that farmers will be able to apply immediately to reduce risks of infections. Lastly, critical issues that surround present procedures used to create “resistant fish” must be be resolved (e.g. carriers, risks, etc.). Currently stamping out may be effective in eradicating the disease. The emerging disease caused by KHV continues to spread. With the economic importance of koi and carp and the vast international movements of koi for the hobby, this disease has the potential for even further spread. The results from our studies form a critical component of a comprehensive program to curtail this emerging pathogen at the local, regional and international levels.

Achievements

Significance of main scientific achievements or innovations.

During the course of the current grant more effective diagnostic tools were developed and key features of the virus and the host-virus relationship critical to the understanding of the epidemiology and control of KHV were examined. The rapid incorporation of the newly developed detection methods and the new knowledge generated on the epidemiology of the agent into regional and international control programs provides a direct testimony of the significance of the achievements from the current grant.

Development of new diagnostic methods – A suite of new diagnostic tools was developed that included 3 PCR assays for detection of KHV DNA in cell culture and fish tissues and an ELISA assay capable of detecting anti-KHV antibodies in the serum of koi and common carp. Ring testing conducted by the CEFAS laboratory in Weymouth, UK, has shown that the TK PCR assay developed in this grant out performed several other PCR methods currently in use for KHV DNA detection and thus it is currently considered the gold standard method for KHV detection.

New features of the virus – Complete genomic sequencing of 3 strains of KHV from Japan, the USA, and Israel revealed a 295 kbp genome containing a 22 kbp terminal direct repeat. Fifteen KHV genes have clear homologs in the distantly related channel catfish virus (*Ictalurid herpesvirus* 1) confirms KHV's proposed place in the family *Herpesviridae*, specifically in the branch with fish and amphibian hosts. The three strains were interpreted as having arisen from a wild type parent encoding 156 unique protein-coding genes, 8 of which are duplicated in the terminal repeat. In each strain, 4 to 7 genes from among a set of 9 are fragmented by frameshifts likely to render the encoded proteins non-functional. Six of the affected genes encode predicted membrane glycoproteins. Frameshifts or other mutations close to the 3'-ends of coding sequences were identified in a further six genes. A proposed association between the apparent loss of some gene functions and high virulence and emergence of the disease provides a basis for further investigations of the molecular epidemiology of the virus. Analysis of DNA

sequence reveals 164 possible ORF's of which only 2 (TK and dUTPase) with demonstrated functions (all other ORF's remain as hypothetical genes). However, 73 out of 164 predicted ORF's were characterized just by motif presence with limited identity to known protein families. The KHV microarray experiments provided the first information on the transcription, including temporal patterns of expression of most ORFs several of which are structural proteins. Many unknown ORF's that show changes in expression patterns under various conditions (temperature, passages no., etc.) may indicate these genes are critical in response to stress, adaptation to changing environmental conditions, and virulence of KHV.

Virus-host relationships/epidemiology/control – The high analytic sensitivity of the quantitative PCR assays developed have been applied to evaluate a key feature of the virus-host interaction, the potential development of the carrier state and virus transmission from such individual fish. Viral DNA has been detected among fish surviving live virus vaccinations or following natural outbreaks in Israel. In these trials, internal organs of common carp (intestine, spleen, kidney and heart) contained between 1 and 10^3 genome equivalents 7 months after experimental vaccination with a wild strain of KHV. Furthermore, the virus was isolated from common carp following cohabitation with carp that were vaccinated. Viral DNA was also detected among most fish surviving natural outbreaks surveyed up to 4 months after exposure. In the US, fish surviving experimental or natural exposures to KHV respond by producing anti-KHV antibodies detected in the serum by ELISA. Although not all fish surviving infection were seropositive, the test provides reliable information on the prior virus exposure at the population level. Currently, a combined application of the ELISA and PCR are viewed as the most powerful control methods available in preventing the spread of KHV. Potential control of KHV in fish production systems by using hybrids of goldfish and common carp demonstrated that hybrids were considerably more resistant than pure common carp or koi to KHV (CyHV-3). In these experimental trials, there was no evidence that goldfish are susceptible hosts to KHV. Also, there was no evidence of KHV replication following bath exposure of several endemic cyprinids species from the western USA.

Agricultural and/or economic impacts of the research findings.

The results from this study have immediate application for the control and management of KHV. New PCR and ELISA tools are currently in use in many areas. Unfortunately, the vaccination procedures with wild type or modified virus compromise programs based upon serologic approaches. Studies on the ecology of the virus also provide insights into management of the virus directly at the farms by decisions on the carrier status and potential transmission between fish groups. This includes the new insights into potential problems with "resistant fish" including establishment of carriers and virus transmission to naïve fish. Currently, only stamping out seems a feasible means to eradicate the disease, particularly when coupled with powerful new detection tools. Currently, the emerging disease caused by KHV continues to spread. The principal pathways for virus spread remain with the current operating procedures of the industry and the hobby. The results from our studies are forming a critical component of what could become a more comprehensive program to curtail this emerging pathogen at the local, regional and international levels.

Details of cooperation.

The TK PCR developed at HU is being used and also tested now in different laboratories worldwide and at Davis. During the duration of the proposal there have been active contacts between the two laboratories mainly concerning the exchanges of methodologies, DNA sequences and analyses. For the field validation of diagnostic tests (PCR), we have exchanged methods and tested in both laboratories independently the developed methods. Likewise, the ELISA serological test developed at Davis was transferred to HU. The evaluation of new sensitive tools such as LC-PCR/TaqMan PCR as well as the TK based PCR is now being done on worldwide basis. The use of our DNA array is being evaluated for in vivo studies in collaboration. Experimental studies on koi in the laboratory have been conducted largely at UC Davis with the assistance of visiting personnel associated with HU. Field studies of the ELISA have been conducted on populations of koi from numerous sources in the vicinity of the UC Davis laboratory.

Appendix (technical information supporting the research findings):

Published papers

Gilad O., S. Yun, F. Zagmutt, C.M. Leutenegger, H. Bercovier and R.P. Hedrick. 2004 Concentrations of a herpes-like virus (KHV) in tissues of experimentally-infected *Cyprinus carpio* koi as assessed by real-time TaqMan PCR. Diseases of Aquatic Organisms 60:179-187.

Bercovier H., Y. Fishman, R. Nahary, S. Sinai, A. Zlotkin, M. Eyngor, O. Gilad, A. Eldar and R.P. Hedrick. 2005. Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis. BMC Microbiology 17:5(1):13.

Adkison M.A., O. Gilad and R.P. Hedrick. 2005. An Enzyme Linked Immunosorbent Assay (ELISA) for Detection of Antibodies to the Koi Herpesvirus (KHV) in the Serum of Koi *Cyprinus carpio koi*. Fish Pathology 40:53-62.

Waltzek T.B., G.O. Kelley, D.M. Stone, K. Way, L. Hanson, H. Fukuda, I. Hirono, T. Aoki, A.J. Davison and R.P. Hedrick. 2005. Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family Herpesviridae. Journal of General Virology 86(6):1659-67.

Hedrick, R.P., O. Gilad, S.C. Yun, T.S. McDowell, T.B. Waltzek, G.O. Kelley and M.A. Adkison. 2005. Initial isolation and characterization of a herpes-like virus (KHV) from koi and common carp. Bulletin of Fisheries Research Agency. Supplement 2:1–7.

Hedrick, R.P., T.B. Watlzek, T.S. McDowell. 2006. Susceptibility of koi carp, common carp, goldfish and goldfish x common carp hybrids to cyprinid herpesvirus-2 and herpesvirus-3. Journal of Aquatic Animal Health 18:26-34.

Aoki T., I. Hirono, K. Kurokawa, H. Fukuda, R. Nahary, A. Eldar , A.J. Davison, T.B. Waltzek, H. Bercovier and R.P. Hedrick. 2007. Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. Journal of Virology 81(10):5058-65.

In press papers

None

Submitted papers

None

In preparation

Nahary R., M. Ravins, M. Eingor, A. Eldar, R.P. Hedrick and H. Bercovier. Transcriptome of Cyprinid herpesvirus 3 (CyHV-3).

Unpublished data

None